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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John C. COX *et al.*

Title: IMMUNOGENIC COMPLEXES AND METHODS RELATING THERETO

Appl. No.: 09/506,011

Filing Date: February 17, 2000

Examiner: ****

Art Unit: 1648

DECLARATION UNDER 37 CFR §1.132

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, John Cox, declare that:

1. I am a citizen of Australia. A copy of my curriculum vitae is an attachment to another declaration of mine, previously submitted and made of record in the above-captioned application ("the application").

2. As I stated before, I am an Intellectual Property Consultant. A major client of mine is CSL, the assignee of the application, which names me as a co-inventor.

3. With reference to a document that also is of record in the application and that is entitled "Preliminary Amendment Under 37 CFR § 1.114," I have reviewed and believe that I understand commentary bearing on the reliance placed by the Patent Office on PCT application WO 98/22135 ("Berglindh").

4. Berglindh describes the preparation of "negatively charged lipids," which, in the presence of Ca^{++}, are said to form negatively charged lipid aggregates or "cochleates." These cochleates can be formed in the presence of an antigen and, hence, can serve as delivery agents in a vaccine.

5. Cochleates may be prepared in the presence or absence of a sterol, typically cholesterol. Berglindh also teaches that various adjuvants may be added to the cochleate formulation. Saponins are mentioned as one category of possible additive. See page 11, lines 5 – 10.

6. The application describes and claims an immunogenic complex that comprises a negatively charged organic complex and a charged antigen. The organic complex itself contains interacting components of a saponin and sterol, and mixing of the organic complex with an antigen leads to formation, by electrostatic interaction, of the immunogenic complex.

7. An immunogenic complex of this sort, as claimed in the application, differs fundamentally from the cochleate aggregates of Berglindh. For example, the formation of cochleates requires the "rolling-up" of a lipid sheet into a spiral roll. Because antigen is present during this process, most of the antigen ends up hidden within the structure, rather than exposed at the surface. For mucosal delivery, which is a goal of Berglindh (for example, see his claim 17), this rolling-up/hidden-antigen feature presents an advantage because the antigen, in principle, will be protected from the hostile mucosal environment. On the other hand, the same feature is a disadvantage for a formulation designed for parenteral delivery, as in the case of the immunogenic complex claimed in the application because the majority of the antigen is not readily presented to antigen presenting cells in its native format.

8. Furthermore, cochleates are structures of several hundred nanometers in diameter and of variable length generally in excess of one micron even after sonication. Sharply contrasting this are the cage-like structures of the claimed immunogenic complexes, which are typically 40 nanometers in size and which are ideal for rapid delivery to lymphoid cells, following parenteral delivery.

9. Finally, the fact that the cochleates of Berglindh are formed in the presence of antigen, as I have noted above, means that the antigen necessarily is exposed to potentially denaturing levels of detergent. By contrast, the immunogenic complexes claimed in the application are the result of mixing antigen with preformed organic complexes, which favors a preservation of native antigen structure.

10. In light of these major structural and functional differences, the following experiments were undertaken, at my direction, to determine what effect, if any, the addition of saponin would have on the cochleates of Berglindh. Because cochleates can form in the absence of antigen, antigen was omitted to simplify experimental procedures. The resultant cochleates were characterized; samples then were mixed with a biologically appropriate quantity of saponin and were recharacterized.

11. The commentary below further details the experimental materials and methodology employed for this purpose.

(A) The following table lists the chemicals employed in the study:

Table 1: Chemicals and relevant chemical information

Chemical	Synonym	Formula, Composition, MW (g/mol)	Other (stored at RT unless stated otherwise)
DPPS	L-α-phosphatidyl-L-serine, or 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-L-serine] sodium salt	$C_{38}H_{73}NO_{10}PNa$ (758.0)	Sigma, Lot #114H0099, –20°C
BPS	brain phosphatidylserine, L-α-phosphatidylserine (Brain, Porcine) sodium salt	$C_{42}H_{79}NO_{10}Pna$ (812.05)	Avanti Polar Lipids, Lot #BPS-713, Prod #830032P, 17/2/05, -20°C
TES	N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid	$C_6H_{15}NO_6S$ (229.2)	Sigma, Lot #39F-5621, Exp.21/12/2010, RT
NOG	N-octyl-β-D-glucopyranoside	(292.4)	Calbiochem, Lot #B23314, 5°C
CH	Cholesterol Super from Wool Grease	(386.66)	Avanti, Lot #CH-41, RFT 9711000076, –70°C
L-histidine	N/A	$C_6H_9N_3O_2$ (155.16)	Sigma (Fluka), Product Code #53319 Lot #423834/1, Exp. N/A, RT
$CaCl_2{\cdot}2H_2O$	N/A	(147.02)	RFT 0404000288 Exp. 14/04/09, RT
NaCl	N/A	(58.44)	#0409000145 (Ajax), exp. 7/9/09, RT
NaOH	N/A	N/A	5 M for pH adjusting, RT
ISP	ISCOPREP® saponin(ISP) Quillaia saponin	pH 6 (8.656 mg/mL ISP*)	Sterile; Stored at –70°C Lot # 81632066

Chemicals stored at –20° and –70°C were thawed at room temperature (RT) for at least 30 minutes, before opening and weighing. ISP was thawed in a water bath at 26 - 28°C for 1 hour, followed by dispensing under clean-room conditions.

(B) Lipids were dissolved in a non-ionic buffer (NOG), before dialysis in Ca^{2+} solution:

(1) 100 mL NOG buffer (2% w/w NOG, 2mM TES, 2mM L-Histidine, 100mM NaCl, pH 8) was prepared by mixing 2 g NOG with 98 g TES/histidine/NaCl buffer and adjusting pH with 5M NaOH.

(2) Lipid (DPPS or BPS) was dissolved in buffer solutions, with or without cholesterol (CH), as follows:

(i) Preparing 2.7 mg/mL DPPS or BPS + 0.3 mg/mL CH

- 3.0 mg of cholesterol was dissolved in 10.0 mL NOG buffer (water bath at 70°C).

- 10.8 mg of DPPS or BPS lipid was then dissolved in 4.0 mL CH/buffer solution (at 35-40°C).

(ii) Preparing 3.0 mg/mL DPPS or BPS lipid.

- 18.0 mg DPPS or BPS lipid was dissolved in 6.0 mL buffer solution (at 35-40°C).

(3) Ca^{2+} dialysis was performed on each solution, according to the following procedure. A Pierce "Slide-A-Lyzer 10K" dialysis cassette (10 000 MW cut off) was immersed in the initial dialysis solution for about 30 seconds, to enable hydration. The dialysis cassette was gently tapped dry, and the lipid/buffer mixture was carefully injected, using a 21 gauge Precision Glide needle and a 5 mL BD Slip Tip Latex Free syringe. Dialysis was performed at room temperature, in four stages: 4 hours and overnight in two separate 3 mM $CaCl_2$-buffered solutions, and then 4 hours and overnight in two separate 6 mM $CaCl_2$-buffered solutions. The buffer for these dialysis solutions was similar to before (2mM TES, 2mM L-Histidine and 100 mM NaCl, at pH 8), except that these did not contain NOG. Each dialysis solution was 1 L in volume and was gently stirred.

(4) The BPS-prepared cochleates were additionally dialyzed with a pH 6.2 BIS buffer, under conditions more suitable for ISP stability. A sample of cochleate mixture was extracted before this dialysis stage.

(5) 0.25 mg/mL ISP was added to a sample of each solution.

(6) The DPPS-prepared cochleates (mixtures designated "C1" and "C2" in Table 2, *infra*) were sonicated briefly, to break up aggregates.

(7) Samples of "neat" cochleates, "sonicated" cochleates and "ISP + cochleates" were extracted for electron-microscopic investigation, the results of which are summarized in the next paragraph.

12. Table 2 summarizes the main observations of the study described above. Figure 1 shows micrographs of a cholesterol-containing cochleate mixture. Figure 2 illustrates the effect of adding 0.25 mg/mL ISP saponin to the same cochleate mixture.

Table 2: Summary of Results

Cochleate Mixture	Components			Observations			
				Visual		EM	
	DPPS	BPS	CH	"neat" cochleate mixture	+ 0.25 mg/mL ISP	"neat" cochleate mixture	+ 0.25 mg/mL ISP
C1	✓	×	✓	Solution was shaken to break up gelatinous mass, particles of up to 2 mm precipitated, supernatant clear after 4 days in cold room; optical microscopy at 10X showed translucent sponge-like structures. More homogeneous after sonication, most particles <1 mm, smaller particles remaining in suspension (some settling over 4 days in cold room).	No immediate effect was observed; fine precipitate after ~2 h (fine ppt remained after 4 days).	"Cochleate-like structures and twisted cylinders" were observed which appeared as "short cochleates" after sonication.	Sonicated cochleates + ISP looked very different in some cases. Considerable disorder, sometimes shorter, disrupted and connected to cage-like structures. "Slightly disrupted short cochleates and adherent structures. Area of 'CLS' material."
C2	✓	×	×	Similar to C1, probably less thick; particles appear more "fluffy". More homogeneous following sonication, most particles in suspension + some flocculate on surface. A few long (~5 mm), white particles were observed on surface following sonication. After 4 days in cold room most particles as flocculate.	Mixture appeared to be slightly cloudier once ISP added; most particles in surface flocculate (note that these large particles produced from sonication were excluded during sampling).	"Long cochleates in tangles." Following sonication, "short and medium cochleates, often in tangles" were observed.	"Short cochleates."
C3	×	✓	✓	White particulate precipitate (particles up to ~ 1 mm) in clear solution. Similar to C1 and C2. No sonication required.	no obvious change	"Cochleates + liposomes."	"Cochleates, lamellar liposomes, membranes with holes related to CLSs, CLS."
C4	×	✓	×	as above	as above	as above	"Cochleates + liposomes."
C3, BIS dialysed	×	✓	✓	as above	as above	as above	"Cochleates, springs, membranes with holes, CLSs with reduced number of holes."
C4, BIS dialysed	×	✓	×	as above	as above	as above	"Cochleates + liposomes."

Cochleates prepared with cholesterol clearly were affected by ISP saponin. In the case of C1, for instance, this was observed with a noticeable precipitate, which formed in 2 hours. The electron micrographs of both C1 and C3 revealed disrupted cochleates and connected, cage-like structures (CLSs) and membranes, which vastly differed in appearance from the "neat" cochleate samples. The BIS dialysis seemed to have no effect. The cochleates prepared using the BPS lipid appeared to be less "gelatinous" (see DPPS-prepared cochleates). This is likely to have resulted from greater mobility of the unsaturated BPS lipid.

Figure 1. Electron micrographs (via negative-staining) of cochleate mixture C1 (DPPS+CH) pre-sonication. (a) Amorphous array of fibrous-like cochleate particles. (b) "Scroll-like" cochleate particles under higher magnification; layers appearing to wrap.

Figure 1a. 23,000 **Figure 1b.** 60,000

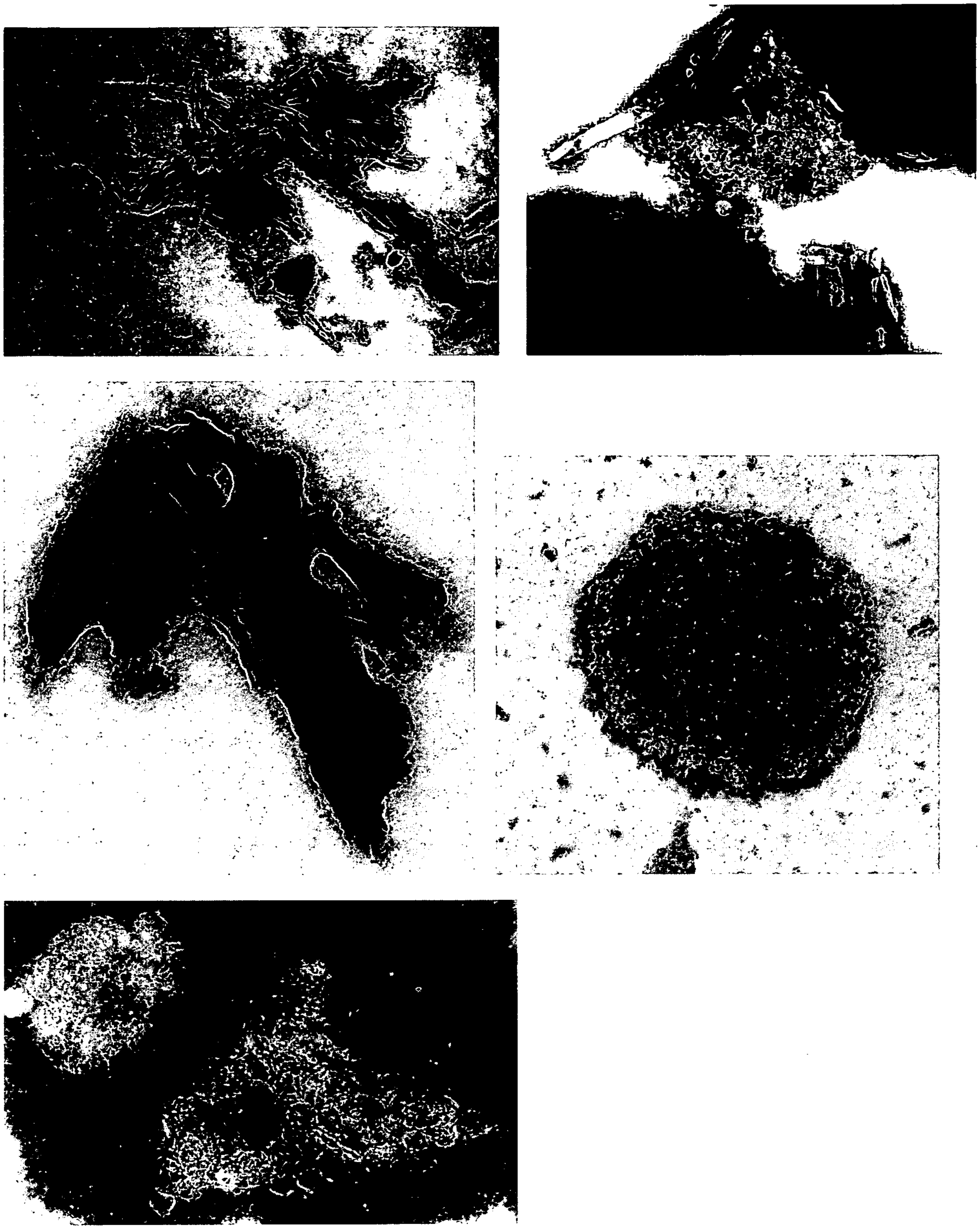

Fig. 2. Electron micrographs (via negative-staining) of cochleate mixture C1 (DPPS+CH), post-sonication and following the addition of 0.25 mg/mL ISP saponin. Cochleate particles appear disrupted and CLSs are evident, often adhering to cochleate particles.

Fig. 2a. 50,000; **Fig. 2b**. 40,000 **Fig. 2c.** 80,000; **Fig. 2d.** 90,000; **Fig. 2e.** 70,000

13. In summary, cochleates made according to Berglindh, absent cholesterol, appeared to be unaffected by addition of saponin. This total absence of change reflects the fact of no interaction between the saponin and cochleates, I believe. Under these conditions, in other words, there was no formation of a complex between saponin and any component of the cochleates. Cochleates made according to Berglindh but in the presence of cholesterol underwent major and variable structural change, as visualized in Figure 2. In my opinion, this indicates that the formulation would not be able to be made in a consistent manner and that the cochleate structures would continue to disorganize over time, such that a formulation thereof would be unstable and would resist adequate characterization.

14. I hereby declare that all the statements made herein of my known knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements are so made punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

12 September 2005
Date

John Cox